wherein hepatocytes and nonparenchymal cells of the co-culture are obtained by perfusion of liver tissue with collagenase and for the population of matrix/hepatic cell clusters of claim 14 obtained by this method, the specification does not reasonably provide enablement for a method of providing a combination of hepatocytes and nonparenchymal cells for co-culturing as claimed, and for obtaining the population of matrix/hepatic cell clusters of claim 14 by another method.

The test for enablement is whether one reasonably skilled in the art could make and use the invention, without undue experimentation, from the disclosure in the patent specification <u>coupled</u> with information known in the art at the time the patent application was filed. *U.S. v. Telectronics Inc.*, 857 F. 2d. 778, 8 USPQ 2d 1217. Furthermore, a patent need not teach, and preferably omits, what is well known in the art. *Hybridtech Inc., v. Monoclonal Antibodies, Inc.* 802 F 2d., 1367, 231 USPQ 81 (Fed. Cir. 1986)

Applicants contend that the present invention is based on the discovery of a novel tissue culture system that provides for long term culture of proliferating hepatocytes that retain hepatic function. Specifically, the invention provides methods for co-culturing of hepatocytes and nonparenchymal cells on a matrix coated with a molecule that promotes cell adhesion in the presence of growth factors. Applicants maintain that the initial methods for obtaining hepatocytes and nonparenchymal cells from liver tissue is not the basis for the present invention because such techniques are well known to those of skill in the art. In this regard the Examiner's, attention is directed to page 14, line 3 through page 15, line 2 of the specification, which discloses that hepatic and nonparenchymal cells can be isolated by a number of different methods, including techniques that are known to those of skill in the art. Such methods include disaggregation of liver tissue mechanically

on enzymatically. Based on this disclosure, the pending claims are fully enabled for the entire scope of the recited subject matter. Therefore, the rejections under 35 U.S.C. §112, first paragraph, should be withdrawn.

2. The Claims are Definite

Claims 1-7, 12 and 14 are rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicants regard as the invention.

According to the Examiner, the term "nonparenchymal cells" is uncertain as to meaning and scope. The specification has not provided a sufficiently definite and precise definition of this negative term to enable one to know when cells used are parenchymal and nonparenchymal.

Applicants assert that contrary to the Examiner's contention, the meaning of the term "nonparenchymal is definite and precise. First, the term nonparenchymal is a known term of art implying any cells of the liver other than hepatocytes. In this regard, the Examiner's attention is directed to Exhibit A which comprises four publication abstracts demonstrating the use of the term "nonparenchymal" to designate non-hepatocyte cells of the liver. Second, the Examiner's attention is directed to page 24, lines 13-16 of the specification, which clearly defines nonparenchymal cells as " the cell pellet isolated from the supernatant of the first low gravity centrifugation used to prepare hepatocytes. This fraction primarily contains cells of Ito, bile duct cells, and endothelial cells."

The last line in claim 1 is unclear as to the relationship of the hepatocytes that retain hepatic function to the hepatocytes cultured in line 2. Applicants have amended claim 1 to indicate that the hepatocytes that retain hepatic function are those co-cultured hepatocytes referred to in line 2.

The Examiner alleges that in claim 14, the meaning and scope of matrix/hepatic cell clusters is uncertain. In particular, it is uncertain as to whether the hepatic cell or matrix or both form the clusters, and the shape that is a "cluster" is uncertain. Applicants have amended claim 14 to delete the term "cluster".

In view of the foregoing amendments to the claims, the rejections under 35 U.S.C. §112, second paragraph, should be withdrawn.

3. The Claimed Invention is Novel

Claims 1-7, 12 and 14 are rejected under 35 U.S.C. § 102(a) as being anticipated by Michalopoulos et al. (Hepatology 1999; "Michalopoulos").

The Examiner maintains that the claims are drawn to a method of generating a hepatic cell culture by co-culturing hepatocytes and nonparenchymal cells in the presence of growth factors and a matrix coated with at least one biologically active molecule that promotes cell adhesion, proliferation or survival under conditions sufficient to allow for the proliferation of the hepatocytes while retaining hepatic function of the hepatocytes. Also claimed (claim 14) is a population of matrix/hepatic cell clusters.

The Examiner alleges that Michalopoulos discloses a method of co-culturing hepatocytes and nonparenchymal cells as claimed. A population of matrix/hepatic cell clusters as required by claim 14 inherently results from the method of Michalopoulos.

In addition, since Michalopoulos contains five additional co-authors who are not inventors, claims 1-7, 12 and 14 are rejected under 35 U.S.C. § 102(f) because the Applicants did not invent the claimed subject matter.

Applicants assert that the claimed invention is not anticipated by Michalopoulos. In this regard, the Examiner's attention is directed to the Declaration of Dr. George K. Michalopolulos and William C. Bowen, submitted herewith, stating that the additional authors listed on the publication, *i.e.*, Valerie F. Zajac, Donna Beer-Stolz, Simon Watkins, Vsevolod Kostrubsky and Steven C. Strom, carried out, under their instructions, specific assays to merely test the features of the novel tissue culture system (¶3 of Declaration). Thus, the rejections under 35 U.S.C. §102 (a) and 102(f) should be withdrawn.

4. <u>The Claimed Invention is Not Obvious</u>

Claims 1, 2, 4-7, 12 and 14 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Mitaka et al. (Hepatology 1999; "Mitaka") in view of Naughton et al. (U.S. 5,624,840; "Naughton") and Vacanti et al. (U.S. 5,759,830; "Vacanti").

Mitaka is alleged to disclose obtaining hepatic cells and nonparenchymal cells from liver tissue and culturing the hepatic cells and nonparenchymal cells together for hepatic organoid reconstruction.

According to the Examiner, Naughton discloses growing stromal cells on a three-dimensional matrix such as made from nylon or polystyrene (col. 8, line 1) which may be coated with collagen (col. 8, line 8) to form a three-dimensional stromal matrix (col. 8, lines 30-40), and then growing hepatocytes on the stromal matrix to form tissue

having liver function (col. 11, lines 54-57). The Examiner maintains that Vacanti discloses growing hepatocytes (col. 6, line 28) in a three-dimensional fibrous scaffold to form tissue having liver function for implanting (col. 5, line 35 to col. 6, line 62, and col. 12, lines 17-47). The fibers of the scaffold may be coated with collagen to enhance cell attachment (col. 10, lines 44-47), and epithelial cells may be attached to the scaffold in combination with the hepatocytes (col. 12, lines 25-27).

The Examiner asserts that it would have been obvious to carry out the culturing of hepatic cells and nonparenchymal cells together as disclosed by Mitaka on a three-dimensional matrix or scaffold as suggested by Naughton and Vacanti to obtain the function of the matrix or scaffold in producing tissue having liver function. Moreover, it would have been obvious to grow hepatocytes directly on the matrix without first forming stromal tissue since it is clear from Vacanti that stromal tissue can be omitted.

Claim 3, which requires that the matrix be in the form of polystyrene beads, is rejected under 35 U.S.C. § 103(a) as being unpatentable over the references as applied to claims 1, 2, 4-7, 12 and 14 above, and further in view of Matsui et al. (U.S. 5,298,615; "Matsui").The Examiner alleges that Matsui discloses that it is standard procedure to culture animal cells on microcarriers such as polystyrene beads coated with collagen (col. 2, lines 10-25).

Applicants respectfully disagree with the Examiner's rejection and submit that the claimed invention is not rendered obvious by the cited references using the objective standard for obviousness under 35 U.S.C. §103. As set forth in *Graham v. Deere*, a finding of obviousness under 35 U.S.C. §103 requires a determination of the scope and content of the prior art, the level of ordinary skill in the art, the differences between the

claimed subject matter and the prior art, and whether the differences are such that the subject matter as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made. *Graham v Deere, Inc.* 383 U.S. 1 (1966).

First, because the January 1999 publication date of Mitaka is within one year of the December 7, 1999 priority date of the present application and given the Declaration of Dr. George K. Michalopolulos and William C. Bowen, submitted herewith, stating that they had submitted a manuscript which included data demonstrating the co-culturing of hepatic cells and nonparenchymal cells prior to the publication date of Mitaka, the reference of Mitaka is unavailable as a prior art reference.

Thus, in the present instance, the proper inquiry is whether Naughton, Vacanti and/or Matsui suggest the claimed methods for generating a hepatic cell culture comprising <u>co-culturing hepatocytes and nonparenchymal cells, in the presence of growth factors and a matrix coated with a molecule that promotes cell adhesion</u>. Clearly the answer to this question is no. Naughton merely discloses growing stromal cells on a three dimensional matrix followed by the growing of hepatocytes on the stromal matrix; Vacanti discloses growing hepatocytes in a three-dimensional fibrous scaffold to form tissue having liver function for implanting; and Matsui discloses that it was known to culture animal cells on microcarriers such as polystyrene beads coated with collagen. Each of the cited references <u>fails</u> to disclose, or suggest, the benefit derived from co-culturing hepatocytes and non-parenchymal cells together on a matrix in the presence of growth factors.

Applicants assert that in the present instance, the Examiner has failed to produce any reference that teaches or suggests the co-culturing of hepatocytes and parenchymal

cells, much less, the co-culturing of such cells on a matrix coated with a factor that promotes adhesion in the presence of growth factors. Therefore, the claimed invention is not obvious, and the rejections under 35 U.S.C. §103 should be withdrawn.

CONCLUSION

Entry of the foregoing amendments and remarks into the file of the above-identified application is respectfully requested. Applicants believe that the invention described and defined by the amended claims is patentable over the rejections of the Examiner. Withdrawal of all rejections and reconsideration of the amended claims is requested. An early allowance is earnestly sought.

Respectfully submitted,

Dated: March 26, 2002

Carmella L. Stephens

Rochelle K. Seide
Patent Office Reg. No. 32,300

Carmella L. Stephens
Patent Office Reg. No. 41,328

BAKER BOTTS L.L.P.
30 Rockefeller Plaza
New York, New York 10112-0228

Attorneys for Applicants
(212) 408-2500

APPENDIX

IN THE CLAIMS:

Please amend the claims to read as follows:

1. (Amended) A method for generating a hepatic cell culture comprising co-culturing hepatocytes and nonparenchymal cells, in the presence of growth factors and a matrix coated with at least one biologically active molecule that promotes cell adhesion, proliferation or survival under conditions sufficient to allow for the proliferation of said hepatocytes that retain hepatic function.

14. (Amended) A population of [matrix/hepatic cell clusters comprising] hepatocytes and nonparenchymal cells associated with a matrix coated with at least one biologically active molecule that promotes cell adhesion, proliferation or survival.

1: Histol Histopathol 1997 Oct;12(4):1069-80

The hepatic perisinusoidal stellate cell.

Kawada N.

Department of Internal Medicine, Osaka City University Medical School, Japan.

Hepatic stellate cell (also referred to as Ito cell, fat-storing cell, perisinusoidal cell, lipocyte) is one of the sinusoid-constituent cells that play multiple roles in liver pathophysiology. Although identification of the stellate cell had taken about 100 years because of the misconception caused by the discoverer von Kupffer, Wake made a great contribution to the "re" discovery of the cell in 1971. Establishment of the isolation of hepatic non-parenchymal cells from rats by Knook has made it possible to uncover the metabolic function of individual cells. Now, the stellate cell function is expanding from a retinol (fat)-storing site to a center of extracellular matrix metabolism and mediator production in the liver. Function as a liver specific pericyte has also been elucidated. Transition of the stellate cells from the vitamin A-storing phenotype to "activated" or "myofibroblastic" cells that produce a large amount of type I collagen and transforming growth factor beta triggers the progress of liver fibrosis in the course of hepatic inflammation. Communication of the stellate cells with the other hepatic constituent cells and invading inflammatory cells is also an important factor that regulates the local pathological reaction. Analysis of cellular and molecular aspects of the stellate cell activation would lead to the establishment of a novel therapeutic strategy against the progress of liver fibrosis in human liver disease.

Publication Types:
- Review
- Review, Academic

PMID: 9302568 [PubMed - indexed for MEDLINE]

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1: Mol Cell Biochem 1988 Sep;83(1):3-14 Related Articles, NEW Books, LinkOut

Non-parenchymal cells as mediators of physiological responses in liver.

Altin JG, Bygrave FL.

Department of Biochemistry, Faculty of Science, Australian National University, Canberra ACT.

Parenchymal cells (hepatocytes) are the sites at which the principal metabolic functions of the liver are located. In the perfused liver, responses (e.g. vasoconstriction and glycogenolysis) to stimulating agents such as zymosan, platelet-activating factor and arachidonic acid, are inhibited by indomethacin and bromophenacyl bromide, inhibitors of cyclo-oxygenase and phospholipase A2, respectively. Since cultured Kupffer and endothelial cells but not hepatocytes, produce eicosanoids, and since eicosanoids and especially prostaglandins induce similar patterns of responses when added directly to the perfused liver, an involvement of these non-parenchymal cells in mediating the above responses is considered likely. We propose that in most situations the responses induced by these stimulating agents are mediated through a combination of pathways that include interaction of the agents directly with hepatocytes or with vasoactive cells (endothelial and/or smooth muscle cells), or interaction of agents initially with non-parenchymal cells to produce and release eicosanoids, which then subsequently interact with hepatocytes or with vasoactive cells.

Publication Types:
- Review
- Review, Academic

PMID: 3065613 [PubMed - indexed for MEDLINE]

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1: Enzyme 1992;46(1-3):155-68

Liver cell heterogeneity: functions of non-parenchymal cells.

Bouwens L, De Bleser P, Vanderkerken K, Geerts B, Wisse E.

Laboratory of Cell Biology and Histology, Free University Brussels, Belgium.

The normal hepatic sinusoid is formed or lined by four different cell types, each with its specific phenotypic characteristics, functions and topography. Endothelial cells constitute the closed lining or wall of the capillary. They contain small fenestrations to allow the free diffusion of substances, but not of particles like chylomicrons, between the blood and the hepatocyte surface. This filtering effect regulates the fat uptake by the liver. Sinusoidal endothelial cells also have a pronounced endocytotic capacity which makes them an important part of the reticuloendothelial system. They are also active in the secretion of bioactive factors and extracellular matrix components of the liver. Recently, a zonal heterogeneity of the endothelial lining has been reported with regard to its filtering capacity (fenestration) and binding capacity for lectins and cells. Kupffer cells are intrasinusoidally located tissue macrophages with a pronounced endocytotic capacity. They are potent mediators of the inflammatory response by the secretion of a variety of bioactive factors and play an important part in the immune defense. A zonal heterogeneity has been established with regard to the endocytotic capacity and cytotoxic function. Pit cells are now known to represent a liver-associated population of large granular lymphocytes. They have the capacity to kill tumor cells and probably also play a role in the antiviral defense of the liver. In addition, pit cells may have a growth-regulatory function of the liver. They are known to be numerically more prominent in the periportal region, as is also the case for Kupffer cells. Fat-storing or Ito cells are present in the perisinusoidal space of Disse and are thought to represent the main hepatic source of extracellular matrix components. They are also the main site of vitamin-A storage. Fat-storing cells are more numerous in the periportal region than in the central region of the hepatic acinus. The periportal cells also store higher amounts of vitamin A. Sinusoidal cells may be considered to represent a functional unit at the border line between the hepatocytes or parenchymal cells and the blood. They participate in various liver functions and liver pathologies and our knowledge about this is growing. The heterogeneity of these cell types and

possible cooperations between them and the hepatocytes may add to our understanding of liver functions.

Publication Types:

- Review
- Review, Academic

PMID: 1289080 [PubMed - indexed for MEDLINE]

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1: Adv Anat Embryol Cell Biol 2001;161:III-XIII, 1-151 Related Articles, NEW Books, LinkOut

Cooperation of liver cells in health and disease.

Kmiec Z.

Medical University of Gdansk, Department of Histology and Immunology, 80211 Gdansk, Poland. zkmiec@amg.gda.pl

The liver lobule is formed by parenchymal cells, i.e., hepatocytes and nonparenchymal cells. In contrast to hepatocytes that occupy almost 80% of the total liver volume and perform the majority of numerous liver functions, nonparenchymal liver cells, which contribute only 6.5% to the liver volume, but 40% to the total number of liver cells, are localized in the sinusoidal compartment of the tissue. The walls of hepatic sinusoid are lined by three different cell types: sinusoidal endothelial cells (SEC), Kupffer cells (KC), and hepatic stellate cells (HSC, formerly known as fat-storing cells, Ito cells, lipocytes, perisinusoidal cells, or vitamin A-rich cells). Additionally, intrahepatic lymphocytes (IHL), including pit cells, i.e., liver-specific natural killer cells, are often present in the sinusoidal lumen. It has been increasingly recognized that both under normal and pathological conditions, many hepatocyte functions are regulated by substances released from neighboring nonparenchymal cells. Liver sinusoidal endothelial cells constitute the lining or wall of the hepatic sinusoid. They perform important filtration function due to the presence of small fenestrations that allow free diffusion of many substances, but not of particles of the size of chylomicrons, between the blood and the hepatocyte surface. SEC show huge endocytic capacity for many ligands including glycoproteins, components of the extracellular matrix (ECM; such as hyaluronate, collagen fragments, fibronectin, or chondroitin sulphate proteoglycan), immune complexes, transferrin and ceruloplasmin. SEC may function as antigen-presenting cells (APC) in the context of both MHC-I and MHC-II restriction with the resulting development of antigen-specific T-cell tolerance. They are also active in the secretion of cytokines, eicosanoids (i.e., prostanoids and leukotrienes), endothelin-1, nitric oxide, and some ECM components. Kupffer cells are intrasinusoidally located tissue macrophages with a pronounced endocytic and phagocytic capacity. They are in constant contact with gut-derived particulate materials and soluble bacterial products so that a subthreshold level of their activation in the normal liver may be

anticipated. Hepatic macrophages secrete potent mediators of the inflammatory response (reactive oxygen species, eicosanoids, nitric oxide, carbon monoxide, TNF-alpha, and other cytokines), and thus control the early phase of liver inflammation, playing an important part in innate immune defense. High exposure of Kupffer cells to bacterial products, especially endotoxin (lipopolysaccharide, LPS), can lead to the intensive production of inflammatory mediators, and ultimately to liver injury. Besides typical macrophage activities, Kupffer cells play an important role in the clearance of senescent and damaged erythrocytes. Liver macrophages modulate immune responses via antigen presentation, suppression of T-cell activation by antigen-presenting sinusoidal endothelial cells via paracrine actions of IL-10, prostanoids, and TNF-alpha, and participation in the development of oral tolerance to bacterial superantigens. Moreover, during liver injury and inflammation, Kupffer cells secrete enzymes and cytokines that may damage hepatocytes, and are active in the remodeling of extracellular matrix. Hepatic stellate cells are present in the perisinusoidal space. They are characterized by abundance of intracytoplasmic fat droplets and the presence of well-branched cytoplasmic processes, which embrace endothelial cells and provide focally a double lining for sinusoid. In the normal liver HSC store vitamin A, control turnover of extracellular matrix, and regulate the contractility of sinusoids. Acute damage to hepatocytes activates transformation of quiescent stellate cells into myofibroblast-like cells that play a key role in the development of inflammatory fibrotic response. Pit cells represent a liver-associated population of large granular lymphocytes, i.e., natural killer (NK) cells. They spontaneously kill a variety of tumor cells in an MHC-unrestricted way, and this antitumor activity may be enhanced by the secretion of interferon-gamma. Besides pit cells, the adult liver contains other subpopulations of lymphocytes such as gamma delta T cells, and both "conventional" and "unconventional" alpha beta T cells, the latter containing liver-specific NK T cells. The development of methods for the isolation and culture of main liver cell types allowed to demonstrate that both nonparenchymal and parenchymal cells secrete tens of mediators that exert multiple paracrine and autocrine actions. Co-culture experiments and analyses of the effects of conditioned media on cultures of another liver cell type have enabled the identification of many substances released from non-parenchymal liver cells that evidently regulate some important functions of neighboring hepatocytes and non-hepatocytes. To the key mediators involved in the intercellular communication in the liver belong prostanoids, nitric oxide, endothelin-1, TNF-alpha, interleukins, and chemokines, many growth factors (TGF-beta, PDGF, IGF-I, HGF), and reactive oxygen species (ROS). Paradoxically, the cooperation of liver cells is better understood under some pathological conditions (i.e., in experimental models of liver injury) than in normal liver due to the possibility of comparing cellular phenotype under in vivo and in vitro conditions with the functions of the injured organ. The regulation of vitamin A metabolism provides an example of the physiological role for cellular cross-talk in the normal liver. The majority (up to 80%) of the total body vitamin A is stored in the liver as long-chain fatty acid esters of retinal, serving as the main source of retinoids that are utilized by all tissues throughout the body. Hepatocytes are directly involved in the uptake from blood of chylomicron remnants, and the synthesis of retinol-binding protein

that transfers retinol to other tissues. However, more than 80% of the liver retinoids are stored in lipid droplets of hepatic stellate cells. HSC are capable of both uptake and release of retinol depending on the body's retinol status. The activity of some major enzymes of vitamin A metabolism have been found to be many times higher per protein basis in stellate cells than in hepatocytes. Despite progress in the understanding of the roles played by these two cell types in hepatic retinoid metabolism, the way in which retinoids move between the parenchymal cells, stellate cells, and blood plasma has not been fully elucidated. Sinusoidal blood flow is, to a great extent, regulated by hepatic stellate cells that can contract due to the presence of smooth muscle alpha-actin. The main vasoactive substances that affect constriction or relaxation of HSC derive both from distant sources and from neighboring hepatocytes (carbon monoxide, leukotrienes), endothelial cells (endothelin, nitric oxide, prostaglandins), Kupffer cells (prostaglandins, NO), and stellate cells themselves (endothelin, NO). The cellular cross-talk reflected by the fine-tuned modulation of sinusoidal contraction becomes disturbed under pathological conditions, such as endotoxemia or liver fibrosis, through the excess synthesis of vasoregulatory compounds and the involvement of additional mediators acting in a paracrine way. The liver is an important source of some growth factors and growth factor-binding proteins. Although hepatocytes synthesize the bulk of insulin-like growth factor I (IGF-I), also other types of nonparenchymal liver cells may produce this peptide. Cell-specific expression of distinct IGF-binding proteins observed in the rat and human liver provides the potential for specific regulation of hepatic IGF-I synthesis not only by growth hormone, insulin, and IGF-I, but also by cytokines released from activated Kupffer (IL-1, TNF-alpha, TGF-beta) or stellate cells (TGF-alpha, TGF-beta). Hepatic stellate cells may affect turnover of hepatocytes through the synthesis of potent positive as well as negative signals such as, respectively, hepatocyte-growth-factor or TGF-beta. Although hepatocytes seem not to produce TGF-beta, a pleiotropic cytokine synthesized and secreted in the latent form by Kupffer and stellate cells, they may contribute to its actions in the liver by the intracellular activation of latent TGF-beta, and secretion of the biologically active isoform. Many mediators that reach the liver during inflammatory processes, such as endotoxins, immune-complexes, anaphylatoxins, and PAF, increase glucose output in the perfused liver, but fail to do so in isolated hepatocytes, acting indirectly via prostaglandins released from Kupffer cells. In the liver, prostaglandins synthesized from arachidonic acid mainly in Kupffer cells in a response to various inflammatory stimuli, modulate hepatic glucose metabolism by increasing glycogenolysis in adjacent hepatocytes. The release of glucose from glycogen supports the increased demand for energetic fuel by the inflammatory cells such as leukocytes, and additionally enables enhanced glucose turnover in sinusoidal endothelial cells and Kupffer cells which is necessary for effective defense of these cells against invading microorganisms and oxidative stress in the liver. Leukotrienes, another oxidation product of arachidonic acid, have vasoconstrictive, cholestatic, and metabolic effects in the liver. A transcellular synthesis of cysteinyl leukotrienes (LTC4, LTD4, and LTE4) functions in the liver: LTA4, an important intermediate, is synthesized in Kupffer cells, taken up by hepatocytes, converted into the potent LTC4, and then released into

extracellular space, acting in a paracrine way on Kupffer and sinusoidal endothelial cells. Thus, hepatocytes are target cells for the action of eicosanoids and the site of their transformation and degradation, but can not directly oxidate arachidonic acid to eicosanoids. (ABSTRACT TRUNCATED)

Publication Types:

- Review
- Review, Academic

PMID: 11729749 [PubMed - indexed for MEDLINE]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Michalopoulos et al.

Serial No.: 09/455,952 Examiner: Naff, D.

Filed: December 7, 1999 Group Art Unit: 1651

For: A NOVEL LONG-TERM THREE DIMENSIONAL TISSUE CULTURE SYSTEM

DECLARATION OF DR. GEORGE K. MICHALOPOULOS AND WILLIAM C.BOWEN

Assistant Commissioner of Patents
Washington, D.C. 20231
SIR:

We, George K. Michalopoulos and William C. Bowen, do declare:

1. We are co-inventors of the invention disclosed in the above identified application.

2. We are co-authors on the publication entitled "Morphogenetic Events in Mixed Cultures of Rat Hepatocytes and Nonparenchymal Cells Maintained in Biological Matrices in the Presence of Hepatocyte Growth Factor and Epidermal Growth Factor" published in January of 1999 (Hepatology 29:90-100).

3. The additional authors listed on the publication, Valerie F. Zajac, Donna Beer-Stolz, Simon Watkins, Vsevolod Kostrubsky and Steven C. Strom, carried out, under our instructions, specific assays to test the features of the novel tissue culture system disclosed in the

above identified patent application. In particular, Ms. Zajac conducted the Northern blot analysis for mRAN expression, using standard methodology; Dr. Beer-Stolz conducted the electron microscopy and immunofluorescence examination of the hepatocytes on roller beads; Dr. Watkins assisted with the interpretation of the immunofluorescence results; Drs. Strom and Kostrubsky provided us with the probes and assisted with the interpretation of the cytochrome P450 mRNA analysis in Figure 12 of the paper.

4. In addition, prior to January 1999, the publication date of Mitaka et al. (Hepatology, 29:111-125), we submitted a manuscript which included data demonstrating the co-culturing of hepatic cells and nonparenchymal cells, derived from liver tissue, in biological matrices. The manuscript was accepted and eventually published as the Michalopoulos et al. publication identified in ¶2 of this declaration. (See, results section, p.93-97 of Michalopoulos et al)

5. We hereby declare further that all statements made herein by our own knowledge are true and that all statements made on information and belief are believed to be true and further that we make these statements with the knowledge that willful false statements and the like are punishable by fine or imprisonment, or both, under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application of any patent issuing therein.

Dated: 3-21-02

George K. Michalopoulos

Dated: 3-21-02

William C. Bowen

Morphogenetic Events in Mixed Cultures of Rat Hepatocytes and Nonparenchymal Cells Maintained in Biological Matrices in the Presence of Hepatocyte Growth Factor and Epidermal Growth Factor

GEORGE K. MICHALOPOULOS, WILLIAM C. BOWEN, VALERIE F. ZAJAC, DONNA BEER-STOLZ, SIMON WATKINS, VSEVOLOD KOSTRUBSKY, AND STEVEN C. STROM

SEE EDITORIAL ON PAGE 288

Hepatocytes were grown in chemically defined hepatocyte growth medium (HGM) containing hepatocyte growth factor (HGF) and epidermal growth factor (EGF) on collagen-coated polystyrene beads in roller bottle cultures, forming clusters of beads, and proliferating hepatocytes and nonparenchymal cells, including fenestrated endothelium-forming vascular structures. Desmin-positive cells surrounded hepatocytes. Collagen types I and III were deposited in a diffuse manner whereas collagen type IV surrounded the clusters of the epithelial cells, forming a basement membrane. When the mixed cell clusters were implanted in Matrigel (Collaborative Research, Bedford, MA), hepatocytes grew in three dimensions, forming plates and ducts. Many single, long plates of hepatocytes were seen, suggesting progressive linear assembly guided by hepatocyte specific structural parameters. HGF, EGF, and transforming growth factor-α (TGF-α) enhance these phenomena. HGF plus EGF elicited maximal response. TGF-β1 suppressed formation of the ducts and plates. Within three months in Matrigel, the cultures established monolayers composed of plates, ducts, and a well-delineated canalicular network. The mixed cultures expressed albumin, A1AT, AFP, transferrin, and CYPIIB1. Following implantation of the cell clusters in Matrigel, there was decreased expression of c-met, urokinase, urokinase receptor, and TGF-β1. Electron microscopy showed differentiated hepatocytes with nearly normal ultrastructure. The proliferating cell nuclear antigen (PCNA) labeling index was high (more than 80%) whereas the Bromo-deoxyaridine labeling index of ongoing DNA synthesis varied from 10% to 15%. These results show that the mixed cultures of proliferating hepatocytes and nonparenchymal cells can reproduce the hallmark structures of hepatic histological architecture while maintaining differentiation and the capacity to proliferate. (HEPATOLOGY 1999;29: 90-100.)

Hepatocytes are highly differentiated cells capable of multiple cycles of proliferation. Recent work with transgenic models indicates that the replicative capacity of nonneoplastic differentiated hepatocytes *in vivo* far exceeds limits established for fibroblasts in culture.[1-4] Serial transplantation of mature hepatocytes was able to rescue mice and repopulate livers for up to eight generations of mice. Normal hepatic architecture was restored when livers were repopulated. The parameters controlling morphogenetic events relevant to the formation of hepatic architecture, however, have not been defined.

Several laboratories have introduced culture techniques that allow hepatocytes in primary culture to grow and/or express complex patterns of differentiation.[5-7] We also have recently established conditions that allow mature hepatocytes to enter into clonal expansion in cell culture.[7] Hepatocytes cultured in chemically defined hepatocyte growth medium (HGM) enter into DNA synthesis in response to polypeptide mitogens, notably epidermal growth factor (EGF), transforming growth factor-α (TGF-α), and hepatocyte growth factor (HGF). These mitogens induce multiple rounds of DNA synthesis and expansion of the cell population. The proliferating cells, however, lose most markers of differentiation while they retain expression of hepatocyte associated transcription factors HNF1, HNF4, and HNF3. These undifferentiated cells become differentiated hepatocytes after addition of matrix derived from the Engelbreth-Holm-Swarm sarcoma (Matrigel [Collaborative Research, Bedford, MA]). The same cells predominantly form ducts when placed in collagen type I gels in the exclusive presence of HGF. In this study, we examined the behavior of these proliferating hepatocytes when forced to grow, not as monolayers but in three dimensions, suspended in a biological matrix. Of the many conditions tested, the best responses were observed from mixed cultures of hepatocytes and nonparenchymal cells growing as clusters of cells and collagen coated plastic beads in roller bottles, subsequently placed in Matrigel. The purpose of this article is to describe the conditions used and the results obtained. The presence of mixed-cell populations and the deposition of matrix in the roller bottle cultures imparts phenotypic stability in terms of differentiated gene expression. The morphogenetic patterns formed in these cultures when placed in Matrigel suggest that hepatocytes alone have innate tendencies to form structures

Abbreviations: HGM, hepatocyte growth medium; EGF, epidermal growth factor; TGF-α, transforming growth factor-α; TGF-β1, transforming growth factor-β1; HGF, hepatocyte growth factor; PBS, phosphate-buffered saline; GAPDH, glyceraldehyde phosphate dehydrogenase; PCNA, proliferating cell nuclear antigen.

From the Departments of Pathology and Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA.

Received March 25, 1998; accepted October 1, 1998.

Supported by NIH Grants CA30241 and CA35373 to G.K.M.

Address reprint requests to: George K. Michalopoulos, M.D., Ph.D., University of Pittsburgh School of Medicine, Pittsburgh, PA 15261. E-mail: michalopoulosgk@msx.upmc.edu; fax: 412-648-9846.

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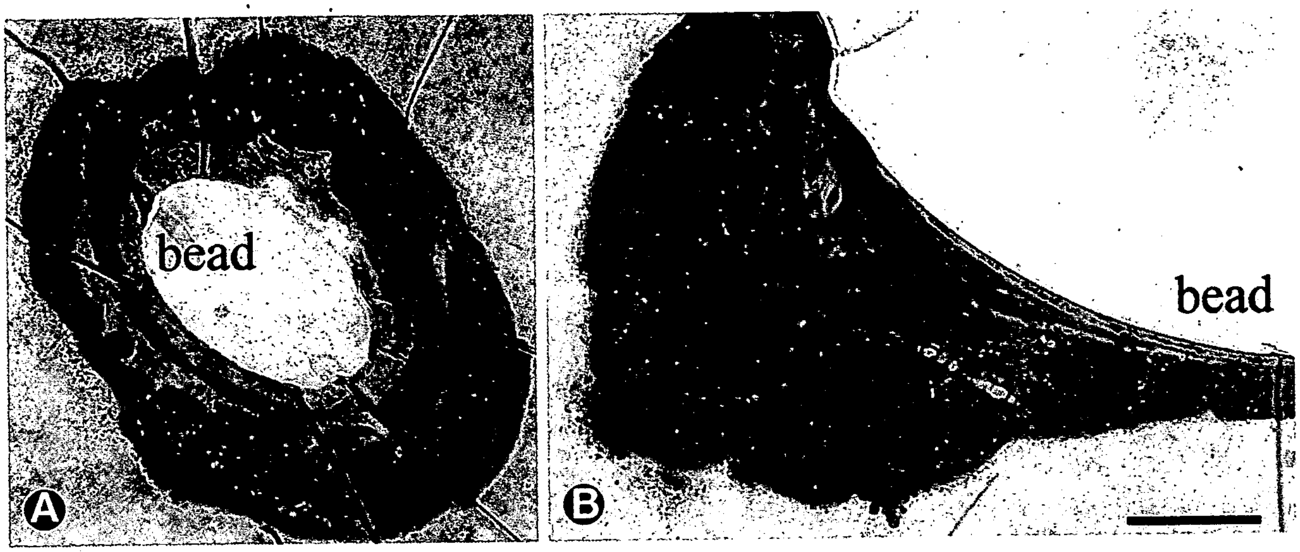


FIG. 1. Thin sections of cells on beads in roller bottle cultures at day 15 after isolation, stained with toluidine blue. (A) The bead is seen as a hollow space in the center of the cell cluster. Gray material around the bead represents dense type-I collagen deposition. The collagen surrounds and embeds connective-tissue–derived nonparenchymal cells. Cells with hepatocyte morphology surround the connective tissue core. (B) Similar as in A. The epithelial cells with hepatocyte morphology form an eccentric growth over a foundation of connective tissue cells. Note the formation of multiple microvilli over the hepatocytes present on the surface.

such as ducts and plates separated by sinusoidal-like spaces, as seen in the organization of the hepatic acinus.

MATERIALS AND METHODS

Animals

Male Fischer 344 rats from Charles River were used for the studies described. All animals were treated according to protocols approved by the animal care institutional review board.

Materials

EGF was obtained from Collaborative Biomedical (Waltham, MA). Collagenase for hepatocyte isolation was obtained from Boehringer Mannheim (Mannheim, Germany). Vitrogen (from Celtrix Labs., Palo Alto, CA) was used for the construction of the collagen gels. General reagents were obtained from Sigma (St. Louis, MO). EGF and Matrigel (Collaborative Research) were purchased from Collaborative Biomedical. HGF used for these studies was the Δ5 variant and was kindly donated by Snow Brand Co. (Toshigi, Japan). Polystyrene beads coated with type I collagen were purchased from SoloHill Inc. (Ann Arbor, MI). Antibody sources: Mouse anti-rat ICAM (CD54) Pharmingen (San Diego, CA) (1:500); rabbit anti-rat collagen I, Chemicon (Temecula, CA) (1:100); rabbit anti-rat collagen III, Chemicon (1:100); Mouse anti-desmin, Dako (Carpenteria, CA) (1:100); Mouse anti-rat monocyte/macrophage (ED-1) Serotec (Raleigh, NC) (1:500); Rabbit anti-rat Collagen IV, generous gift from Dr A. Martinez-Hernandez (1:100).

Isolation and Culture of Hepatocytes

Rat Hepatocytes. Rat hepatocytes were isolated by an adaptation of Seglen's calcium two-step collagenase perfusion technique[8] as previously described from our laboratory.[9] Typically, a 3% contamination with nonparenchymal cells is seen in this isolate.

Nonparenchymal Cell Fraction. Nonparenchymal cell fraction was defined as the cell pellet isolated from the supernatant of the first low-gravity centrifugation used to prepare hepatocytes. This fraction primarily contains cells of Ito, bile duct cells, and endothelial cells. Small hepatocytes are also present in this fraction, typically comprising 5% of the cells.

Roller Bottle Cultures

Freshly isolated hepatocytes were added to roller bottles (850 cm^2 surface) obtained from Falcon (Franklin Lakes, NJ). Each bottle

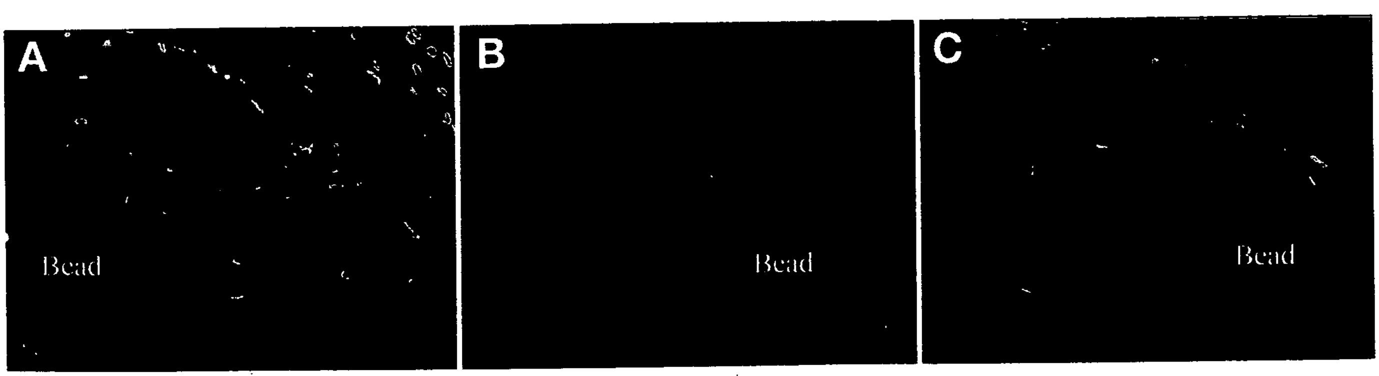
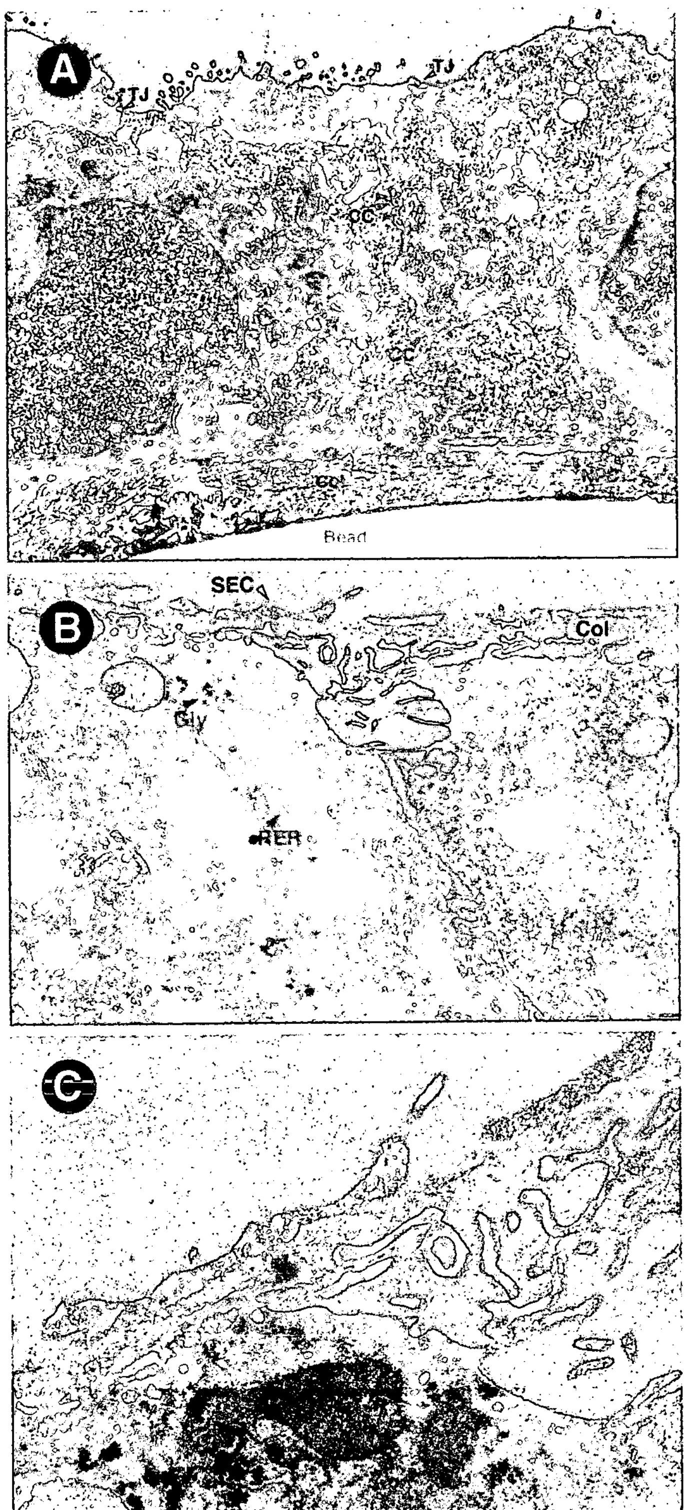


FIG. 2. Matrix deposition in Stage 1 roller bottle cultures. A, B, and C show depositions of collagen types I, III, and IV respectively. Collagen types I and III are deposited as broad bands surrounding the beads. Collagen type IV often formed basement membrane structures surrounding hepatocytes arranged in acinar or ductal configurations. Matrix is stained red whereas nuclei of the adjacent cells are stained blue. Visualization by immunofluorescence microscopy.

contained 18.7 × 10^6 beads and 210 × 10^6 freshly isolated hepatocytes in 250 mL of HGM medium supplemented with HGF (20 ng/mL) and EGF (10 ng/mL). The bottles were rotated at a rate of 2.5 rotations per minute and kept in an incubator maintained at 37°C, saturated humidity, and 5% CO_2. The viability of the cultures was assessed by periodic sampling. The samples were directly observed under a phase contrast microscope as well as stained with methyl tetrazolium to assess viability.



Cultures of Beads in Matrigel

The bead clusters containing cells were isolated from suspensions obtained from the roller bottle cultures. Enrichment for clusters was obtained by allowing for 2 minutes of unit gravity sedimentation. The bead and cell clusters were mixed with Matrigel (Collaborative Research). Bead clusters with cells were allowed to settle whereas beads without cells stayed mostly in suspension. The supernatant was aspirated leaving the clusters in the bottom of the tube. The process was repeated three times. Clusters suspended in medium were mixed with Matrigel at a volume ratio of 1:4 (medium plus beads: Matrigel). Approximately 50 to 100 bead clusters were randomly embedded in Matrigel.

Composition of the HGM

HGM was prepared as previously described.[7] DMEM medium powder, HEPES, glutamine, and antibiotics were purchased from GIBCO/BRL (Grand Island, NY). ITS mixture (Insulin, Transferrin, Selenium) was purchased from Boehringer Mannheim. All other additives were cell-culture grade (Sigma). Unless otherwise indicated for specific experiments, the basal HGM consisted of DMEM supplemented with purified bovine albumin (2.0 g/L), glucose (2.0 g/L), galactose (2.0 g/L), ornithine (0.1 g/L), proline (0.030 g/L), nicotinamide (0.305 g/L), $ZnCl_2$ (0.544 mg/L), $ZnSO_4{:}7H_2O$ (0.750 mg/L), $CuSO_4{:}5H_2O$ (0.20 mg/L), $MnSO_4$ (0.025 mg/L), glutamine (5.0 mmol/L), and dexamethasone (10^{-7} mol/L). Penicillin and streptomycin were added to the basal HGM at 100 mg/L and 100 μg/L, respectively. The mixed basal HGM was sterilized by filtration through a 0.22-μm low-protein–binding filter system, stored at 4°C, and used within 4 weeks. ITS 1.0 g/L, (rh-insulin 5.0 mg/L, human transferrin 5.0 mg/L [30% diferric iron saturated], selenium 5.0 μg/L) was added after filtration immediately before use. The growth factors, as required, were added to HGM fresh at the specified concentrations every time the medium was changed.

Transmission Electron Microscopy

Samples for transmission electron microscopy were washed once in PBS with 1 mmol/L $MgCl_2$, 0.5 mmol/L $CaCl_2$, then fixed overnight at 4°C in 2.5% glutaraldehyde in PBS. Samples were washed three times with PBS then postfixed in 1% OsO4, 1% $KFe(CN)_6$ in PBS for 1 hour at room temperature. Samples were washed three times in PBS, then dehydrated through graded series (30%-100%) of ethanol. Following three changes of 100% ethanol, samples were infiltrated with several changes of Polybed 812 resin (Polysciences, Warrington, PA) at room temperature, a change overnight at 4°C, then a final change, in the case of cells grown on monolayers, where Beem capsules, filled with resin, were inserted on top of areas of interest. Resin was hardened overnight at 37°C, then for 2 additional days at 65°C. While the resin was still warm, Beem capsules were pulled from the dish and analyzed to ensure that the

FIG. 3. Electron microscopy of cultures at Stage 1, (Roller bottle). (A) Low magnification view of hepatocytes growing on beads, before addition of Matrigel (Collaborative Research). Hepatocytes form a continuous multilayer or monolayer culture around the beads and display circuitous, interdigitated cell-cell contacts within the abluminal membrane. Canalicular structures (CC) and tight junctions (TJ) are seen. A 1-micron thick layer of fibrillar collagen (Col) is evident between the hepatocytes' abluminal membranes and the polystyrene bead. A nonparenchymal cell (NPC) is also seen within the fibrillar collagen layer. *Bar* = 1 mmol/L. (B) Another view of the cytoplasmic features of hepatocytes at stage 1 (Magnification, 4,000×). Sinusoidal endothelial cells (SEC) are forming a layer of fenestrated endothelium. Fibrillar collagen (Col) and multiple microvilli are seen under the endothelial layer, with a morphology similar to that seen in the space of Disse. Glycogen (Gly) and lamellae of rough endoplasmic reticulum (RER) are seen in the cytoplasm of the adjacent hepatocytes. (C) Higher magnification of B (10,000×) showing the fenestrae of the endothelial layer. Collagen fibrils are seen in the interrupted cytoplasmic continuity of the endothelial cell at the site of the formation of the fenestra.

cells did not remain on the dish. In some cases monolayers were re-embedded to obtain cross sections. Thick sections (300 μm), obtained using a Reichert (Vienna, Austria) ultramicrotome fitted with a diamond knife, were heated onto glass slides, stained with 1% Toluidine Blue, and rinsed with water. Ultrathin sections (60 nm) were collected on Formvar-coated (Fullam, Schenectady, NY) grids and stained with 2% uranyl acetate in 50% methanol for 10 minutes, then 1% lead citrate for 7 minutes. Sections were analyzed and photographed on a JEOL JEM 1210 transmission electron microscope at 80 kV.

Immunofluorescence Microscopy

Samples from roller-bottle cultures were fixed in 2% paraformaldehyde and 0.01% glutaraldehyde in PBS for 1 hour. Liver seeds were then stabilized by dipping them in 3% gelatin in PBS, then refixing them in the above fixative for an additional 15 minutes. Samples were incubated in 2.3 mol/L sucrose in PBS at 4°C overnight. Samples were mounted on screw stubs and snap-frozen in liquid nitrogen. Five hundred nanometer-thick frozen sections were cut on a FCS Ultracut Microtome (Reichert) fitted with a cryokit. Sections were attached to glass slides by adsorbed Cell-Tak (Collaborative Biomedical). Sections were washed in 0.5% BSA, 0.15% glycine in PBS (PBG buffer) three times to remove sucrose, then blocked with 5% goat serum in PBG buffer for 30 minutes. Sections were then stained with various antibodies (see later) in PBG buffer for 1 hour at room temperature, washed three times in PBG buffer then stained with Cy3-conjugated (goat antirabbit or antimouse) secondary antibodies (Jackson Immunolabs, Bar Harbor, ME) for 1 hour. Sections were washed three times with PBG buffer, then once in PBS. Nuclei were stained with 0.1 mg/mL Hoechst (bisBenzimide) for 30 seconds, washed twice with PBS, then mounted on slides with use of gelvatol (23 g polyvinyl alcohol 2000, 50 mL glycerol, 0.1% sodium azide to 100 mL PBS), and viewed on an Olympus Provis epifluorescence microscope (Olympus America, Melville, NY) also equipped for differential interference microscopy.

Analysis of Gene Expression by Northern Blots

Extraction of Total RNA and Messenger RNA From Cultures. Total RNA was extracted by use of RNAzol B (BioTECX, Houston, TX). RNA extraction from roller-bottle cultures was performed by washing bead-cell clusters in phosphate buffered saline and further digestion of the clusters by adding an equal volume of Trypsin-Ethylenediaminetetraacetic acid (GIBCO-BRL) to the bead-cell suspension. The mixture was shaken at 37°C for 10 minutes. The bead-cell clusters were further washed in S+M buffer at 4°C three times. The bead-cell pellet was mixed with three volumes of RNAzol and purified according to the manufacturer's guidelines.

RNA was extracted from Matrigel (Collaborative Research)-embedded beads by vortexing using 2.0 mL of RNAzol B (BioTECX) per 1 mL of beads in Matrigel and purified per the manufacturer's guidelines. RNA concentration and purity were determined by routine spectrophotometry. Size separation of 20 μg RNA per lane was completed on denaturing 1% agarose gels and transferred to nylon membranes (Amersham, Piscataway, NJ) by the capillary method. After cross-linking under ultraviolet light, membranes were hybridized overnight with specific complementary DNAs (as indicated in Figure 12) that had been labeled with [α-^{32}P]dCTP using an Amersham random primer kit. Membranes were subsequently washed under high stringency conditions and exposed to R film (photographic film) (Kodak, NY) for 1 to 3 days. Quantification of the RNA hybridization bands was performed by laser densitometry.

Sources of Complementary DNA Probes

EGF-R (rat) originated from Dr. Sheldon Earp, University of North Carolina (at Chapel Hill); acidic fibroblast growth factor receptor from American Type Culture Collection (catalog number 78222); acidic fibroblast growth factor receptor from American Type Culture Collection (catalog number 65796); urokinase plasminogen activator originated from Dr. Jay Degen, University of Cincinnati; cytochrome IIB1 from Dr. Steve Strom (University of Pittsburgh); complementary DNAs for albumin, α-fetoprotein were generated by Dr. Joe Locker (University of Pittsburgh).

RESULTS

Morphogenetic Events in Cultures of Different Stages

Stage 1: Cultures of Hepatocytes on Beads in Roller Bottles. Collagen-coated polystyrene beads, as described in Materials and Methods, were placed in roller bottles at a ratio of 18.7 × 10^6 beads to 210 × 10^6 freshly isolated hepatocytes. HGF and EGF were added as standard supplements in the HGM medium of the roller bottle cultures. Cells attached to the beads and, within 2 to 3 weeks, formed clusters of beads bound together with mesenchymal cells surrounded by layers of epithelial cells. The mesenchymal cells concentrate toward the center of the cluster and surround the individual beads (Fig. 1A and B). They are associated with heavy deposition of type I and type III collagen immediately against the surface of the bead (Fig. 2). The collagen bundles surround the mesenchymal cells. Collagen type IV was seen as a thin rim forming a basement membrane surrounding only acinar structures of epithelial cells. The epithelial cells grow outside of the mesenchymal cells and symmetrically surround the

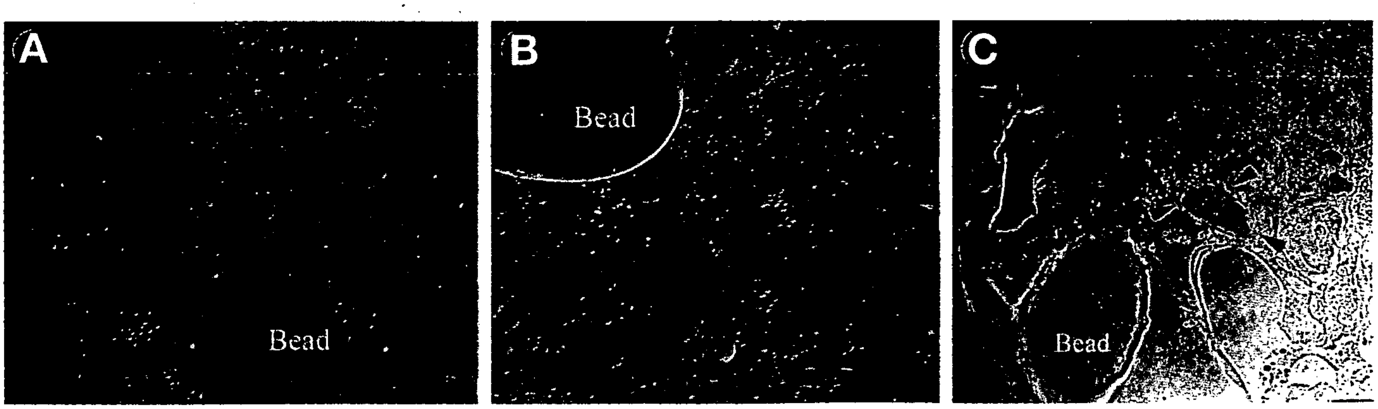


FIG. 4. Stains for macrophages, endothelial cells, and desmin-positive cells in Stage 1 roller bottle cultures. Visualization by differential interference microscopy. Positive immunohistochemistry is shown as red (*complete arrows*) whereas nuclei of cells are stained blue (*truncated arrows*). (A) Macrophages staining positive for ED-1 antigen. Note the "foamy" cytoplasm characteristic of macrophages in some of the cells. (B) Desmin-positive cells. (C) Structures of endothelial cells staining positive for ICAM1 antigen. One of the endothelial cells contains a nucleus at the field of the image (*complete arrow*).

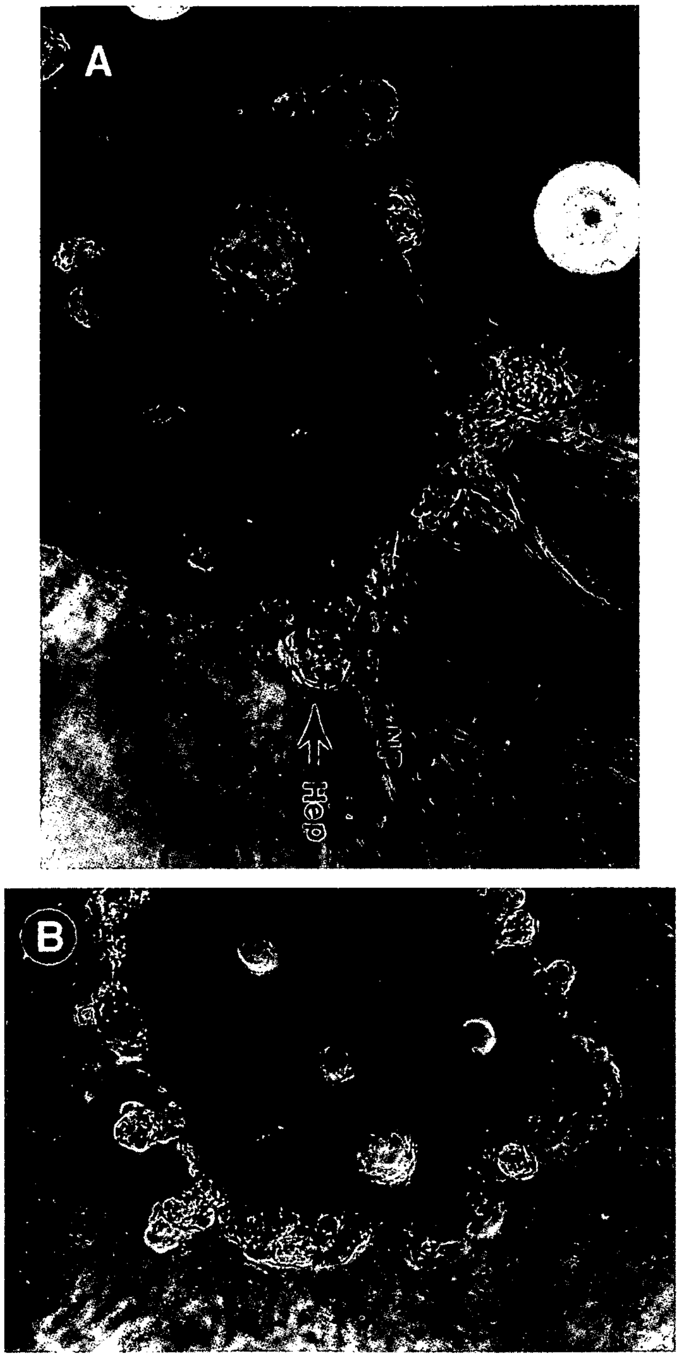


FIG. 5. Migration of cell populations from bead clusters after placement in Matrigel (Collaborative Research). Phase contrast microscopy. (A) Nonparenchymal cells (NP) migrate first and spread by attaching to the substratum. Occasional buddings of epithelial cells are seen at a higher focus plane (Hep). Some (*arrow*) appear to contain a duct. Culture at 1 week in Matrigel. Magnification, 200×. (B) Multiple buddings of epithelial cells migrate out of the bead clusters at different planes and in all directions. Culture at 20 days in Matrigel. Magnification, 200×.

beads or make eccentric projections. The epithelial cells have characteristics of small mature hepatocytes, as shown by electron microscopy. They contain multiple mitochondria and minimal rough endoplasmic reticulum (Fig. 3). Mature bile canaliculi containing microvilli as defined by junctional complexes were occasionally seen. Most often, they appeared as spaces surrounded by hepatocytes and containing micro-

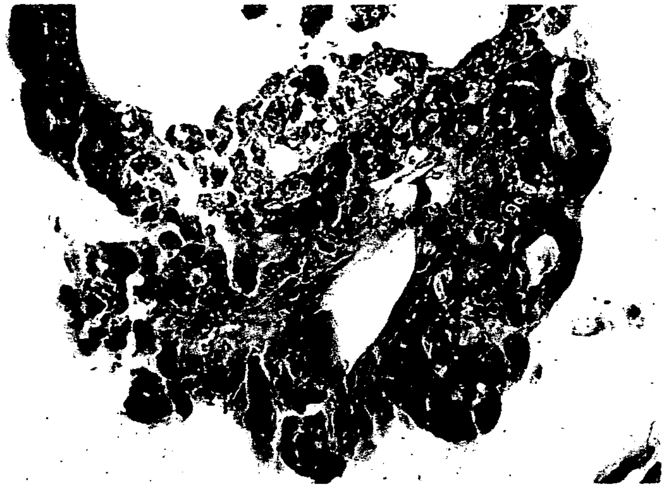
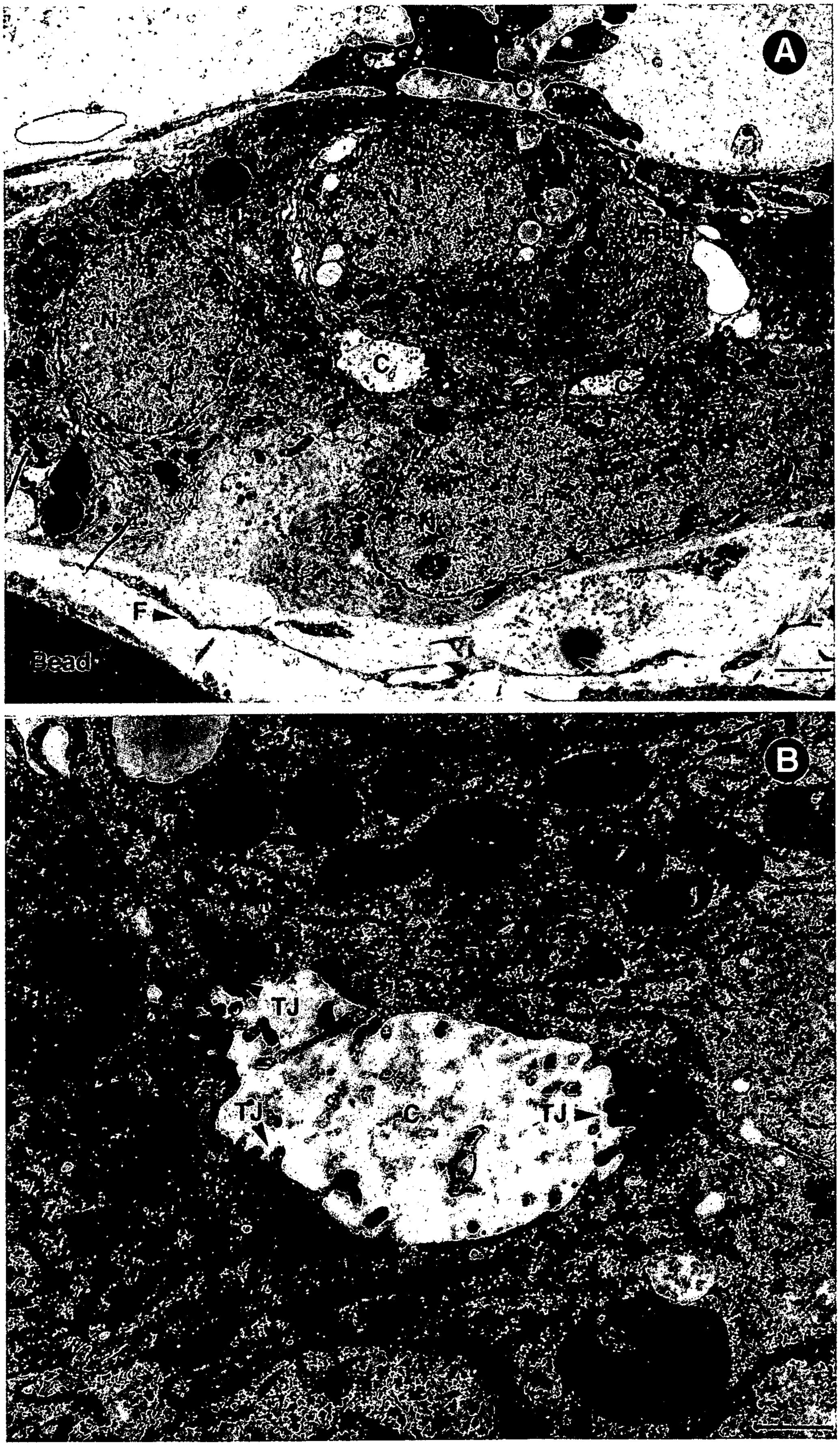


FIG. 6. Histology of the epithelial cell buddings in Matrigel (Collaborative Research) at Stage 2 cultures at day 20 in Matrigel. Epithelial cells with hepatocyte morphology (see Fig. 8) are surrounding the central bead core and are arranged in sheets and ducts. Connective tissue deposition is also present underlying the epithelial cell structures. Hematoxylin eosin stain. Magnification, 200×.

villi. The junctional complexes were not as clearly defined as after placement in Matrigel (Collaborative Research) (see later). Those cells that are on the surface of the clusters have visible microvilli, whereas those toward the interior do not. The epithelial cells form multiple cell layers from the mesenchymal cell layer of the cluster to the surface. The cytoplasmic details of the epithelial cells in the clusters are shown in Fig. 3B and C. Multiple lamellae of rough endoplasmic reticulum and glycogen deposition is seen. Notable is the occasional formation of fenestrated endothelium surrounding the hepatocytes. The PCNA labeling index of the epithelial cells exceeded 70% in all clusters. The BRdU labeling index of epithelial cells varied from 10% to 15% in different clusters. The number of nonparenchymal cells varied from one cluster to another. Figure 4 shows desmin-positive mesenchymal cells, presumably derived from stellate cells contaminating the original hepatocyte preparation,[10] interspersed between the epithelial cells. Approximately 15% to 20% of the cells at this stage seem to belong to this category. ICAM1-positive endothelial cells are also seen in Fig. 4, occasionally forming ICAM1-positive luminal structures. Overall, less than 2% of the cells at this stage stained positive for this antibody. Macrophages, identified as ED-1–positive cells, are seen only in sporadic clusters, representing less than 0.1% of the total cell population.

Stage 2: Cultures in the First 3 Weeks After Implantation in Matrigel. Clusters of beads with the mixed cell populations were placed in Matrigel (Collaborative Research) as described in Materials and Methods. This resulted in a series of cell

FIG. 7. (A) Low power electron micrograph of an acinar structure formed from the bead cluster. Evident are the duct-like canalicular structures (C) in the center of the acinar structure. Cells contain extensive RER and numerous mitochondria. A thick, but less electron dense layer of extracellular matrix than that observed for the pre-Matrigel bead is seen between the hepatocytes and the bead, with several fibroblastic (F) type cells residing in the matrix. Bar = 2 mm. (B) High power micrograph of the canalicular structure seen in A. Readily obvious are three extensive tight junctional areas (TJ), desmosomes, RER, Golgi elements, and Mt, mitochondria. *Bar* = 500 nm.


A
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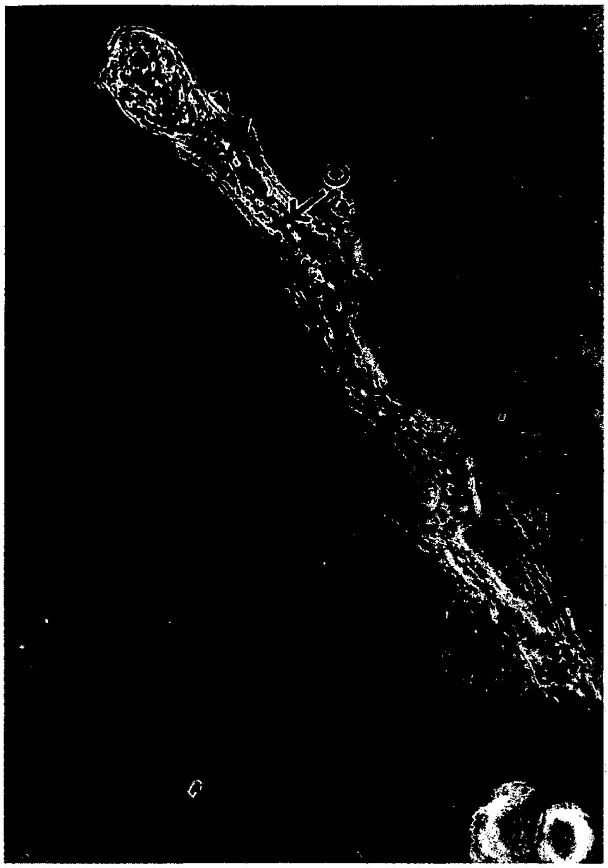


FIG. 8. Formation of plates by hepatocytes at Day 20 in Matrigel (Collaborative Research). Notice the prominent canalicular network (bright canals, *arrows*) along the middle of the plate.

migrations. Mesenchymal cells with stellate shape migrated out of the beads first at about day 4 to 5 and in many instances formed a mat surrounding the beads (Fig. 5A). Protrusions with rounded contours, appearing as buds, were seen extending randomly in all directions from the bead clusters at about day 7 to 10. Some of them (approximately 30%) appeared to contain ducts. The typical appearance of these cultures is shown in Fig. 5B. Sections of these bud structures stained with hematoxylin and eosin are shown in Fig. 6. The buds consisted primarily of hepatocytes arranged in acinar structures or in sheets. Electron microscopy (Fig. 7) showed enhanced cytoplasmic differentiation of hepatocytes compared with cells in the roller bottle. Hepatocytes in the buds contained abundant lamellae of rough endoplasmic reticulum, glycogen, and canaliculi with complete junctional complexes. The latter features are not seen in the hepatocytes before implantation in Matrigel. In most cultures, several long plates, 1 to 2 hepatocytes in width and 10 to 20 hepatocytes in length (Fig. 8), were seen. These structures averaged about 20 to 30 per plate, with plates of different length extending from most clusters. The plates typically developed into areas of the substratum that were free of other cell types. There were no visible nonparenchymal cells underlying or surrounding these plates. A typically demarcated and fully developed canalicular network was seen along the entire length of the plates. Many of these single plates contained ducts at the end. IL6 (10 ng/mL) added to the cultures augmented the number of duct structures and caused formation of ducts along the plates or in the monolayer patches of hepatocytes. TGF-β1 (at 0.5 ng/mL) inhibited formation of all structures that developed from epithelial cells (buds, plates, and ducts) though migration of the nonparenchymal cells was not inhibited. The full spectrum of changes was seen in the presence of HGF plus EGF. Cultures maintained in HGF or EGF alone showed fewer and more-limited changes per cluster compared with those with both growth factors. The extensive budding of the epithelial cells was associated with cell proliferation as judged by staining for PCNA. The numbers of labeled hepatocytes in the Matrigel ranged from 40% to 80% of epithelial cells per cluster, with considerable variation seen from one site to the next or among clusters. The BRdU labeling index, indicating active DNA synthesis, varied from 10% to 15% per cluster. Desmin-positive cells were seen interspersed and surrounding the hepatocytes. Type IV collagen was seen often as a thin rim surrounding acinar structures of hepatocytes. Slight staining was seen for type I and stronger staining for type III collagen (Fig. 9).

Stage 3: Long-Term Cultures in Matrigel. Long-term follow-up showed that HGF or EGF added separately was not sufficient to maintain prolonged viability of the epithelial cells. By 3 months, no epithelial cells were present in cultures maintained in HGF or EGF alone, or in control cultures without the addition of growth factors. In cultures maintained with combined HGF plus EGF, large monolayer patches of hepatocytes ranging from 2 to 10 mm in diameter were seen (Fig. 10). These structures appear at the rate of 2 to 4 patches per plate. These patches had a cytoarchitecture of striking similarity to sections of the liver acinus. Single or double hepatocyte plates were seen extending in a linear or convoluted manner. Complete canalicular networks developed throughout the entire length of each of the plates. The plates were separated by spaces that, though resembling the sinusoidal spaces seen in the liver lobules, did not contain any cells. Occasional ducts were also present in random locations along the plate structures. Electron microscopy (Fig. 11) showed typical hepatocyte morphology with most features typically present in hepatocytes, including glycogen, abundant rough endoplasmic reticulum, microbodies, and bile canaliculi with mature junctional complexes.

Gene Expression Changes in Cultures at Stages 1 and 2

The expression of several genes was examined in cultures at stages 1 and 2. Monolayers at stage 3 were not available in sufficient numbers for RNA preparation. Figure 12 compares expression of several genes in hepatocytes and nonparenchymal cells immediately after isolation from liver, cells from roller bottle cultures at day 13, cells from roller bottle cultures at day 25, and cell-bead clusters at 12 days after implantation in Matrigel (Collaborative Research) (day 25 after cell isolation). The first and last lanes show expression of the same genes respectively in hepatocytes and the nonparenchymal cell fraction, immediately after isolation from the rat liver. (Please note that several hepatocyte-associated genes are expressed in this fraction as a result of contamination by small hepatocytes, as discussed in Materials and Methods.) Though Matrigel-enhanced expression of α-fetoprotein, cultures in the roller bottles and in Matrigel maintained high expression of albumin. EGF-receptor expression decreased in culture, whereas HGF-receptor expression was maintained in roller bottles and in Matrigel, though

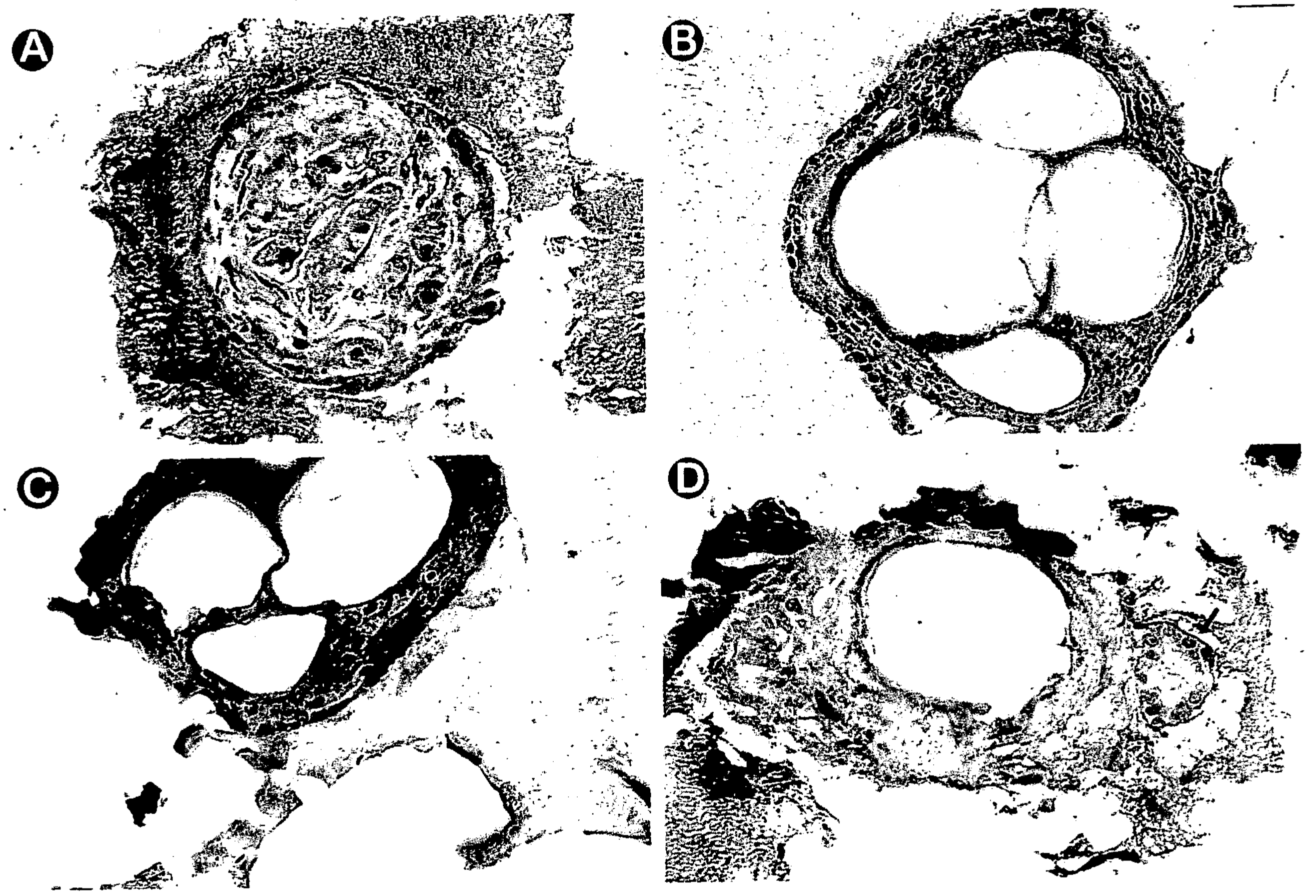


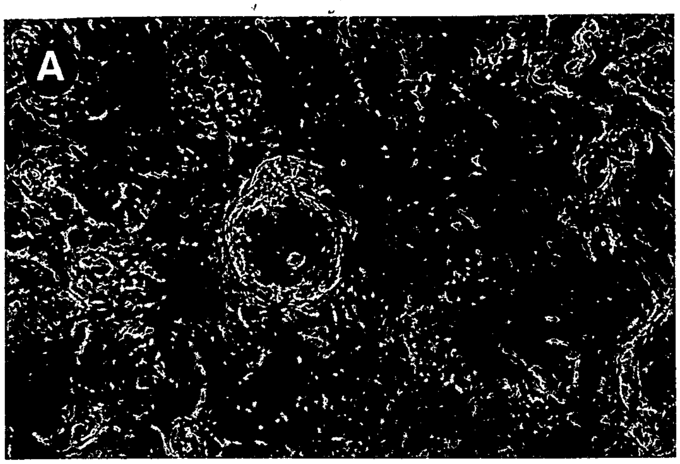
FIG. 9. Cellular and matrix immunohistochemistry in Stage 2 cultures in Matrigel (Collaborative Research). Staining by immunoperoxidase. A, B, C, and D show stains for desmin, Collagen types I, III, and IV respectively. Desmin-positive stellate cells are interspersed in close proximity to the hepatocytes. Collagen type III shows the strongest immunohistochemical response. Collagen type IV often formed basement membrane structures surrounding hepatocytes arranged in acinar or ductal configurations (*arrow*).

Matrigel caused a decrease in *c-met* expression. CYPB1 expression decreased gradually in the roller bottle cultures but was restored after addition of Matrigel. TGF-β1 expression, derived from the nonparenchymal cells present in the mixed cultures, was pronounced in the roller bottle cultures at stage 1 but suppressed by Matrigel in stage 2 cultures. The same was true for urokinase plasminogen activator and its receptor urokinase plasminogen activator-R. Expression of transferrin and α-1 antitrypsin was also enhanced at stage 2. A separate study was conducted to evaluate induction of cytochrome P450 species in stage 1 cultures. Induction of cytochrome P450 species CYP1A, CYP3A, CYP2B1/2 was seen in response to 3′ Methyl-cholanthrene, Dexamethasone, and Phenobarbital, respectively (data not shown).

DISCUSSION

Differentiation of hepatocytes in culture is dependent on the surrounding matrix, the chemical environment of the cell culture medium, and the presence of specific cytokines. Several previous studies have examined the differentiation and proliferation of hepatocytes in monolayers[5-7] or spheroid cultures.[11] The system described here shows that mixed cultures of hepatocytes and nonparenchymal cells derived from liver maintain a remarkable degree of phenotypic stability in the mobile environment of roller bottle cultures. Typically, differentiation features such as induction of cytochrome P450 species and preservation of cellular morphology are lost early on,[7] unless hepatocytes are maintained in the presence of biomatrix gels such as Matrigel (Collaborative Research). Given the multiple examples with hepatocyte monolayer cultures, in which presence of matrix plays a defining role for hepatocyte differentiation, it is very likely that the phenotypic stability of hepatocyte functions in this system is dependent on the matrix synthesized by the nonparenchymal cells. This matrix directly surrounds hepatocytes arranged in acinar structures, as with collagen type IV (see Figs. 2 and 9) or provides a broader structural support as with collagen types I and III. Of interest is the relative abundance of the types of collagen seen in these studies. Collagens type I and III were most abundant whereas collagen type IV was present only in selected sites. This was seen in both Stage I and Stage 2 cultures. The proportions of the collagen species in these cultures parallels those seen in adult rat liver.[12]

The present study also shows that cells derived from mature hepatocytes can interact to develop many organotypic structures occurring in three dimensions during liver development, such as ducts, plates, and spaces between plates reminiscent of sinusoidal areas. The cells in these cultures are of two types, epithelial and mesenchymal. The epithelial cells are similar to those we have previously described in monolayer cultures maintained under the same conditions. We called these cells "hepatoblasts" because they are derived from hepatocytes by dedifferentiation and can redifferentiate to



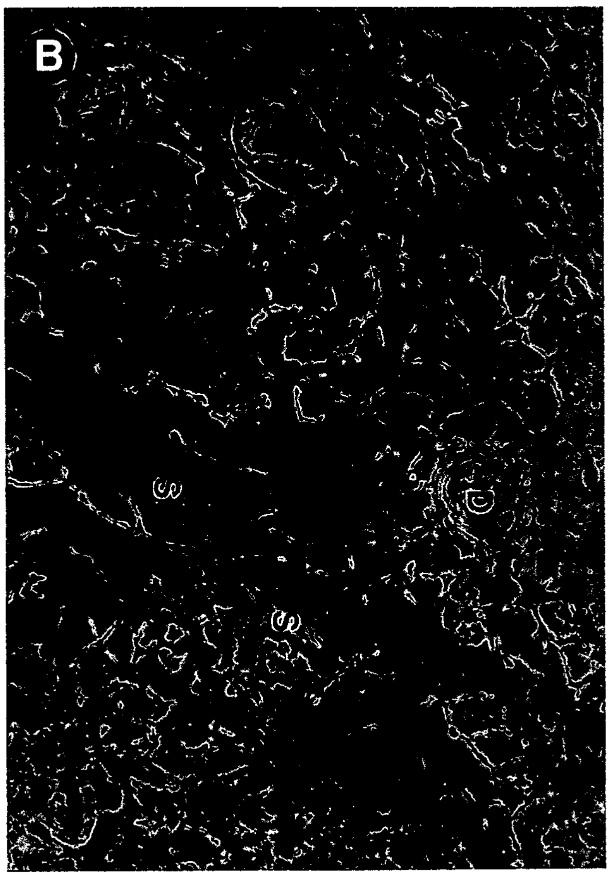


FIG. 10. Phase contrast microscopy of monolayers developing at 2 to 3 months in Matrigel (Collaborative Research) (Stage 3 cultures) in the presence of HGF and EGF. (A) Magnification, 100×. (B) Magnification, 200×. Notice the extensive canalicular network (*bright lines ramifying with short branches* along the hepatocyte plates), the pseudo-sinusoidal spaces (S), and the duct-like structures (D).

form hepatocytes and cells organized in ductular structures. Given the fact that we use the same medium (HGM) and growth factors (HGF and EGF) as in the original study,[7] we believe that these cells are the ones giving rise to the hepatocytes described in this study. The nonparenchymal cells observed in the current study however must derive from the contaminant 3% to 5% of cell population present from hepatocyte isolation.[8] In standard monolayer cultures most of the nonparenchymal cells have been characterized as derived from stellate cells.[10] The stellate morphology of the nonparenchymal cells seen in our cultures and the positive immunohistochemistry for desmin also suggest the same derivation. Lipid droplets of different sizes were seen in these cells in Stage-2 cultures. On the other hand, endothelial cells in variable numbers are also present, as shown in Fig. 4. Given the microarchitecture of the cultures and the presence of Matrigel (Collaborative Research), the percentage of the Ito cells to endothelial cells cannot be precisely determined. They appear to be less than 15% of the overall population in Stage 1 and 2 cultures. Endothelial cells are much less than that (<1%), although sufficient for forming a fenestrated endothelium in conjunction with well-differentiated hepatocytes. Though occasional macrophage derived cells were also noted, their number did not exceed 2% of the total number of cells in these cultures. The mechanisms driving the formation of fenestrated endothelial cells have been studied by other investigators.[13] The current model may allow definition of the fenestrated endothelial cell phenotype in relation to liver.

The presence of ducts and plates in Stage 2 shows that hepatocytes can form either of these structures while maintaining their phenotype. This is also seen *in vivo* in conditions such as fulminant hepatic failure in humans.[14] Of interest were many single plates present in Stage 2, from 5 to 20 cells in length. Because these plates emerged from compact cell-bead clusters of Stage 1, it is likely that they evolved by gradual elongation caused by cell proliferation of either a basal cellular unit at the beginning of the plate or from all cells along the plate. The latter alternative seems more likely because PCNA-labeled cells were randomly placed along the plates. The finding of plate structures in culture suggests that this principle of forming plates is innate to hepatocytes and perhaps responsible for the linear assembly of hepatocytes within the liver acinus *in vivo*. In histological cross sections of normal liver, hepatocytes are arranged in plates surrounded by sinusoids. It is not clear whether this is also true in three dimensions or whether hepatocytes are in reality arranged in two-dimensional "walls," presenting as plates in cross sections. All plates, which were observed in this study, appeared attached to the substrate but were not in direct contact with nonparenchymal cells. The guiding principle leading to progressive elongation of the plates in a monodimensional linear pathway is not clear. Bile canaliculi were present along the entire length of the plates. These structures are fairly rigid, surrounded by a triple barrier of tight junctions, desmosomes, and gap junctions running along the entire length of the canaliculus. A sheath of contractile elements surrounds the above junctions and contributes to the maintenance of bile flow.[15] We are proposing that the canaliculi establish rigid linear guides in the form of pipes, which link the progeny of cell division of single hepatocytes in such a way that a simple linear canaliculus is favored, free from angular distortion. This would tend to favor formation of linear plates traversed by a canalicular network from end to end as seen in our cultures. It is quite possible that other structures on hepatocyte membranes besides canaliculi contribute to the formation of the linear plates. Such structures may be any of the multiple differentiated domains of hepatocyte membranes. It is important to underscore however that no mesenchymal cells appeared to surround or otherwise guide the plates. Hepatocytes by themselves provided the

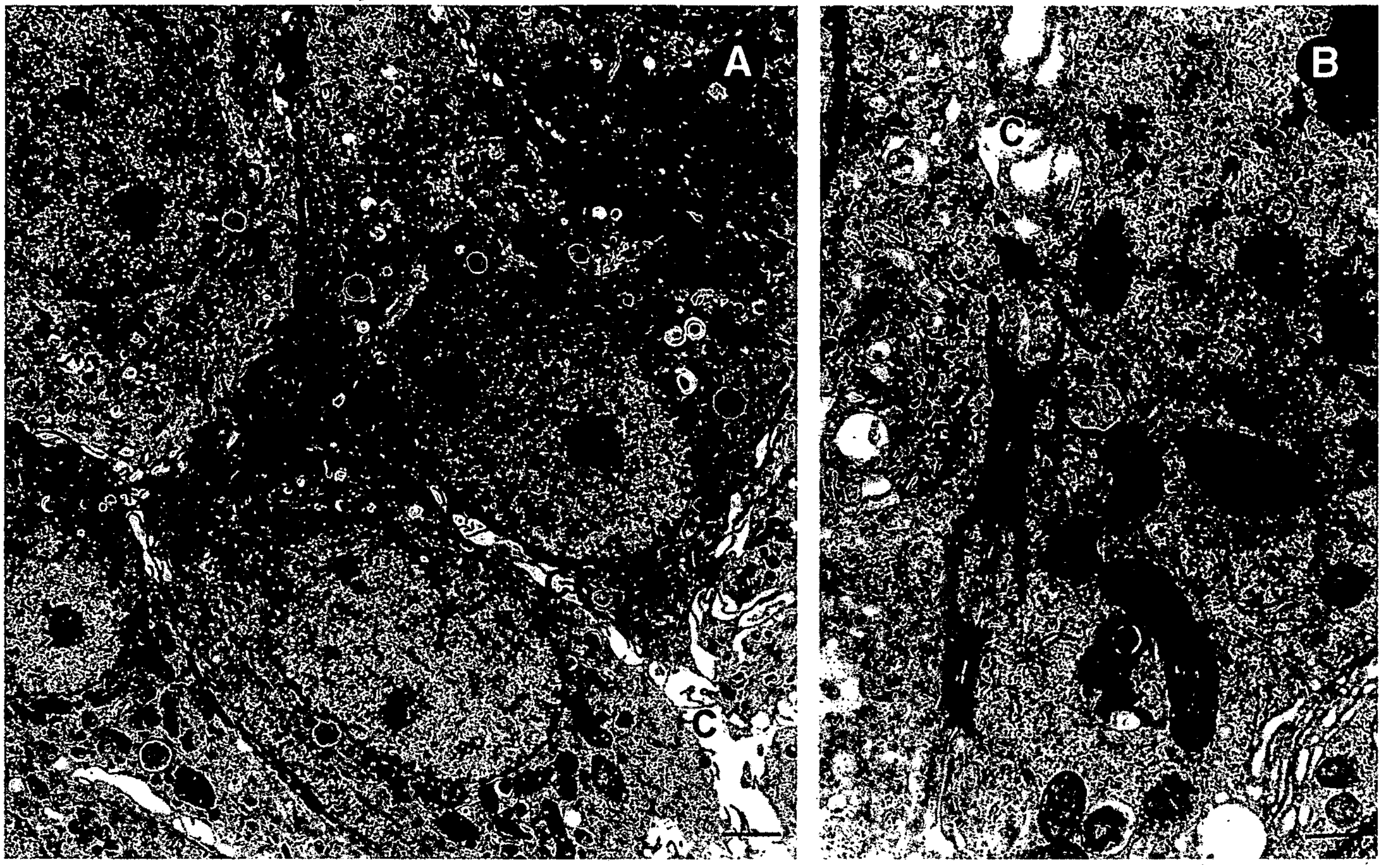


FIG. 11. (A) A low power (2,000×) electron micrograph of hepatocytes in Stage 3 cultures. Notice the longitudinal section of the extensive canalicular network (with microvilli and desmosomes) surrounding the individual hepatocytes. (B) Higher power view (10,000×) showing detailed cytoplasmic features. Rough endoplasmic reticulum, mitochondria, and Golgi network elements are seen in the individual hepatocytes.

structural units that assembled these structures. This does not imply that nonparenchymal cells did not produce cytokines or matrix that affected this process; it suggests however that nonparenchymal cells are not directly involved in providing the structural components for the plate assembly. The model proposed from our results in Stage 3 cultures suggests that hepatocytes are responsible for the formation of the main microarchitecture of the acinus and that the stromal cells (Ito cells, sinusoidal endothelial cells, and Kupffer cells) fit within the architectural parameters established by the hepatocyte assembly.

The degree of differentiation seen in the roller bottle cultures was greater than previously observed in monolayer cultures under the same conditions. Roller bottle cultures as described in this study are easy to set up and maintain stability for long periods of time, exceeding 2 months (data not shown). The stability of the cells on beads may be a result of the presence of multiple cell types, each providing matrix and cytokines for the adjacent cells. The stability of the cultures, the high degree of differentiation and the facility of harvesting make these cultures suitable for use in design of bio-artificial liver systems. Most of the systems used today rely on short-term cultures or established cell lines.[16] The former lack longevity of function whereas the latter generally do not provide enough function to be of real use. Mass cultures as described for the Stage 1 in this presentation may have significant advantages in that they appear to overcome both of these deficiencies.

The combination of cell proliferation and differentiation seen in the cultures at Stage 2 with Matrigel (Collaborative Research) is unusual. Hepatocytes, as is the case with many other cell types, maintain differentiation but do not prolifer-

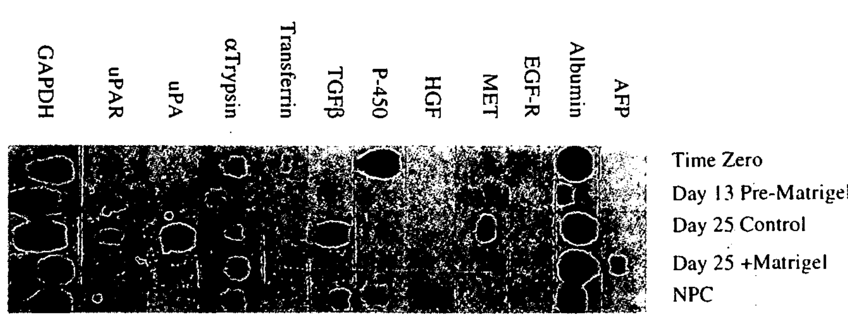


FIG. 12. Expression of several genes in hepatocytes immediately after isolation (Time zero), cells in roller bottle at day 13, cells in roller bottle at day 25, cells in Matrigel (Collaborative Research) cultures at day 25 (12 days after placement in Matrigel at Day 13), and nonparenchymal hepatic cell fraction (5% parenchymal hepatocyte contamination) immediately after isolation. Expression of GAPDH is used as a normalizing parameter. For comments, see Results.

ate in Matrigel.[17] This has been variably ascribed to the presence of cytokines in Matrigel that inhibit cell proliferation, such as TGF-β1.[18] Indeed, commercial preparations of Matrigel depleted of TGF-β1 have been made commercially available to overcome such antiproliferative effects.[19] Our results indicate that TGF-β1 is not responsible for inhibition of hepatocyte proliferation by Matrigel. In fact, more than 70% of hepatocytes were positive for PCNA in the presence of Matrigel, whereas addition of TGF-β1 inhibited morphogenetic events and cell proliferation. If the low levels of TGF-β1 present in Matrigel were responsible for the observed inhibition of hepatocyte proliferation seen in other systems, this does not appear to be the case here. Alternatively, it is also possible that hepatocytes in this system are for unknown reasons more resistant to TGF-β1 and require higher concentrations than those present in Matrigel to cease cell proliferation. It should be noted that high expression of TGF-β1 was seen in the roller bottle cultures presumably by the nonparenchymal cells. We do not know whether this generates active TGF-β1 or the latent form or whether the concentration of TGF-β1 in the high volume of the medium of the roller bottle cultures is sufficient to be effective.

The morphogenetic events of the epithelial cells were enhanced when both EGF and HGF were added to the cultures. Stage 3 cultures were not seen unless both EGF and HGF were present. We have previously shown that HGF and EGF have synergistic effects on the proliferation of monolayers of hepatocytes in HGM medium.[7] The synergy between the two growth factors appears to also extend to events related to hepatocyte differentiation. A recent publication[20] showed that activation of EGF receptor by enhanced expression of TGF-α causes activation of the HGF receptor as well, suggesting a cross talk between the two different receptors. This cross talk may be responsible for the synergy between HGF and EGF observed in this study.

In summary, hepatocytes in long term roller bottle cultures (Stage 1) enter into a stable phenotype in terms of morphology and gene expression, probably because of the deposition of matrix by the associated nonparenchymal cells. The latter also form complex structures as evidenced by the fenestrated endothelium. Stage 2 cultures undergo complex morphogenetic transformations forming ducts and plates in the presence of HGF, EGF, and Matrigel (Collaborative Research). The growth in three dimensions forms ducts and sheets of mature hepatocytes surrounded by nonparenchymal cells. Proliferation and cell differentiation was very high in these cultures. Long plates of hepatocytes unattached to any other cell types were also seen, suggesting that hepatocytes alone have the innate tendency to form these structures. The capacity of hepatocytes in these Stage 2 cultures to undergo both sustained proliferation and maintain differentiation (in contrast to findings with cultures of pure hepatocyte monolayers) suggests that hepatic nonparenchymal cells are required for hepatocytes to be able to exercise both of these functions.

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